In vitro and in vivo investigation of the gastrointestinal behavior of simvastatin.

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ABSTRACT

Simvastatin (SV) is marketed as a lactone ester prodrug which is hydrolyzed to the active simvastatin hydroxyacid (SVA). SV is characterized by a low solubility and undergoes extensive first-pass metabolism. In this study, the influence of the upper gastrointestinal environment on the intraluminal behavior of simvastatin was investigated by a series of in vitro experiments. Dissolution, stability and two-stage dissolution tests were performed using simulated and human gastrointestinal fluids.

The dissolution studies revealed a relatively slow dissolution of SV as well as conversion of SV to SVA. The hydrolysis of SV was further examined and stability studies indicated a faster conversion in gastric fluids than in intestinal fluids. These isolated phenomena were then confirmed by the more integrative two-stage dissolution studies.

To estimate the predictive value of the in vitro tests, an additional in vivo study was performed in which the gastrointestinal concentration-time profiles also revealed a slow dissolution of SV and faster degradation of SV to SVA in the stomach than in the intestinal tract. However, the plasma concentrations of SV and SVA did not directly correlate with the observed gastrointestinal concentrations, suggesting that gut wall and hepatic metabolism have a greater impact on systemic exposure of SV than the intraluminal interconversion between SV and SVA.

1. INTRODUCTION

Nowadays, most drug candidates suffer from low aqueous solubility or low intestinal permeability, resulting in poor oral bioavailability. (Lipinski et al., 2001) The development of prodrugs exhibiting improved solubility and/or permeability has been successful as a strategy to counter these challenges. (Jarkko et al., 2008) It has been demonstrated, however, that premature intraluminal hydrolysis mediated by hydrolyzing enzymes present in the intestinal fluids may significantly alter the intestinal absorption of a prodrug. (Brouwers et al., 2007); (Stappaerts et al., 2015) It has for instance been shown that intraluminal degradation of the ester prodrug tenofovir disoproxil fumarate takes place in vivo, illustrating that the esterases present in the intestinal fluids may undermine the intended enhanced permeability. In another recently described study, the hydrolyzing capacity of intestinal fluids was revealed to be an effective trigger causing abiraterone supersaturation upon administration of the ester prodrug abiraterone acetate; esterase-mediated hydrolysis was shown to be beneficial for the intestinal absorption of abiraterone. (Stappaerts et al., 2015) It is clear that the intraluminal behavior of ester prodrugs can be diverse and may have significant repercussions on intestinal drug absorption. In vitro stability testing in biorelevant media containing hydrolyzing enzymes is clearly an important step in assessing the feasibility of a prodrug approach.

Statins are indispensable in the primary and secondary prevention of cardiovascular diseases worldwide. (Scandinavian Simvastatin Survival Study Group, 1994) This class of drugs competitively inhibits the rate-limiting step of cholesterol biosynthesis, mediated by 3-hydroxy-3-methylglutaryl-coenzym A reductase, decreasing cholesterol neogenesis. (Vickers et al., 1990b) Statins are on the market as either the active hydroxyacid form (atorvastatin, fluvastatin, rosuvastatin and pravastatin) or as lactone (a cyclic ester) prodrug (simvastatin (SV) and lovastatin). (Li et al., 2011); (Lennernäs and Fager, 1997) SV is hydrolyzed to the active metabolite simvastatin hydroxyacid (SVA) by esterases, paraoxonases and by non-enzymatic hydrolysis (Figure 1). (Pedersen and Tobert, 2004)(Prueksaritanont, 2002)

Simvastatin has a low oral bioavailability of less than 5%, which may be attributed to low intestinal uptake and extensive first-pass metabolism. (Kato, 2008) Solubilized simvastatin, on the other hand, is well absorbed from the gastrointestinal tract. (Mauro, 1993) This was confirmed by a high apical to basolateral transport across Caco-2 cell layers for both SV and SVA. (Li et al., 2011) Hepatic uptake of SV occurs through a combination of passive and active transport mediated by the liver-specific isoforms of the Organic Anion Transporting Polypeptide (OATP) family, i.e. OATP 1B1/1B3. (Thompson et al., 2013) In the liver, SV is metabolized via various pathways including acid/lactone interconversion. Both SV and SVA are substrates for CYP3A4. (Figure 1) (Vickers et al., 1990b); (Pedersen and Tobert, 2004); (Prueksaritanont, 2002); (Bottorff and Hansten, 2000); (Cheng et al., 1994) Biliary excretion is found to be the major route of elimination of the SV metabolites. (Vickers et al., 1990a)

In view of our recent findings on the intraluminal stability of several ester prodrugs, the aim of this study was to gain more insight into the intraluminal behavior of the cyclic ester SV. To reach this goal, several *in vitro* experiments were designed involving the use of biorelevant media such as simulated and human gastric and intestinal fluids. In addition, more complex in vitro models were used including two compartmental set-ups to further increase the in vivo similarity. In addition, a clinical study was performed to investigate (1) for the first time the *in vivo* intraluminal behavior of SV and (2) the predictive value of the performed *in vitro* tests.

2. MATERIALS AND METHODS

2.1. Chemicals

Simvastatin (SV) and simvastatin acid (SVA) were both obtained from Sigma-Aldrich (St. Louis, MO) as well as rosuvastatin (RSV), monobasic potassium phosphate monohydrate ($KH_2PO_4.H_2O$), bis-4-nitrophenylphosphate and pancreatin from porcine pancreas (powder, suitable for cell culture, 4x USP specifications). Dimethylsulfoxide (DMSO) and tetrabutylammonium sulfate were obtained from Acros-Organics (Geel, Belgium). Acetic acid was purchased from Chem-lab (Zedelgem, Belgium). Acetonitrile was purchased from Fisher Scientific (Leicestershire, UK). Simulated intestinal fluid (SIF) powder was purchased from Biorelevant (Croydon, UK). Methanol and sodium acetate trihydrate were purchased from VWR International (Leuven, Belgium). Water was purified with a Maxima system (Elga Ltd., High Wycombe Bucks, UK). For the measurements of the pH, a Portamess 911 pH-meter (Knick GmbH & Company, Berlin, Germany) was used. All stock solutions were prepared in DMSO.

2.2. Stabilization mixture

Precautions were taken to guarantee stability of the samples before the analysis. It is known that the stability of simvastatin decreases with increasing temperature whereas sufficient stability has been reported in a pH range of 3 to 6. (Álvarez-Lueje et al., 2005); (Di and Kerns, 2009) All samples were immediately diluted 1/100 in a stabilization mixture (pH 3.5), consisting of MeOH:0.02N HCl (50:50) containing 400 µM of the esterase inhibitor bis-4-nitrophenylphosphate. The stability of the ester prodrug was confirmed in this stabilization mixture.

2.3. Media

Fasted state simulated intestinal fluid (FaSSIF) and fasted state simulated gastric fluid (FaSSGF) were made according to the manufacturer's preparation protocol (Biorelevant®, Croydon, UK). FaSSIF was prepared by dissolving SIF powder (2.24 mg/mL) in a phosphate buffer (pH 6.5). To provide the

simulated fluids with hydrolyzing capacity, FaSSIF was supplemented with pancreatin (10 mg/mL) as described by Borde et al. (Borde et al., 2012) After vortex mixing, this suspension was centrifuged (2.880 *g*) and the supernatant was used for the stability study. FaSSGF was prepared by dissolving SIF powder (0.06 mg/mL) in an HCl/NaCl solution (pH 1.6). For the two-stage dissolution experiment double concentrated FaSSIF (pH 7.5) was prepared in order to obtain taurocholate and lecithin concentrations of 3 mM and and 0.75 mM, respectively, and a pH of 6.5 upon 1:1 dilution with FaSSGF.

Fasted state human gastric (FaHGF) and intestinal (FaHIF) fluids were aspirated from four healthy volunteers (two males, two females) aged between 23 and 27 years. The study was approved by the Committee of Medical Ethics of the University Hospitals Leuven (S53791), Belgium and the procedure followed the tenets of the Declaration of Helsinki. HIF were collected from the duodenum (D2–D3) with a double-lumen polyvinyl catheter [Salem Sump Tube 14 Ch (external diameter 4.7 mm), Sherwood Medical, Petit Rechain, Belgium]. Samples were collected every 10 min for 120 min and kept on ice until pooling. Pooled samples were made by combining equal volumes of the aspirates from all four volunteers. Pooled HIF were stored at −30°C until further use. A similar approach was used for the collection of human gastric fluids (HGF) in which a double-lumen polyvinyl catheter was positioned in the stomach. The pH of the pooled gastric and intestinal fluids of the fasted state amounted to 1.8 and 6.84, respectively.

2.4. In silico profiling

MarvinSketch (ChemAxon, Budapest, Hungary) was used to determine key physicochemical properties including dissociation constant (pKa) and aqueous solubility.

2.5. In vitro studies

2.5.1. Dissolution study

The dissolution behavior of SV and SVA in gastric fluids, FaHGF (pH 1.72) and FaSSGF (pH 1.6), was determined by adding 0.13 mg to 1 mL of FaSSGF. An amount of 0.13 mg reflects the intake of 1 tablet of 40 mg together with 250 mL water, further diluted in 50 mL of residual stomach fluids. The experiments were performed in Eppendorf tubes that were shaken horizontally (175 rpm) at 37 °C for 2 h. Samples were taken at predetermined time points: 5, 15, 30, 60, 90 and 120 min.

The dissolution behavior of SV and SVA in FaHIF and FaSSIF was determined by adding 1 mg of SV or SVA to 1 mL of each medium. The experiments were performed in Eppendorf tubes that were shaken horizontally (175 rpm) at 37 °C for 4 h. Samples were taken at predetermined time points: 10, 20, 40, 60, 120, 180 and 240 min.

To determine the concentrations of SV and SVA, the samples were centrifuged (10 min, 20.817 g) and the supernatant was diluted 1/100 in the stabilization mixture before analysis. To determine the total amount per volume of SV and SVA, the samples were immediately diluted 1/100 in the stabilization mixture, followed by a centrifugation step (10 min, 20.817 g). The supernatant was used for analysis. All experiments were performed in triplicate.

2.5.2. Stability study

To explore the stability of SV and SVA in gastric fluids, 10 µM of SV and SVA was spiked into simulated and human gastric fluids: FaSSGF and FaHGF. Samples were taken at predetermined time points: 0, 60, 90 and 120 min. In addition to the gastric fluids, the stability of SV and SVA was also studied in simulated and human intestinal fluids. Three different media with increasing levels of complexity in the following order: FaSSIF, FaSSIF supplemented with pancreatin (10 mg/mL) and FaHIF. Similar to the gastric fluids, 10 µM of SV or SVA was spiked into the different media. Samples were taken at predetermined time points: 0, 60, 120, 180 and 240 min. All samples were diluted immediately 1/100 in the stabilization mixture. The samples were centrifuged (20.817 *g*) and the supernatant was used for analysis. All experiments were performed in triplicate.

2.5.3. Two-stage dissolution testing

Two-stage dissolution tests were performed using simulated and human gastric and intestinal fluids. For the simulated fluids, one tablet of Zocor® (simvastatin, 40 mg) was added to 50 mL of FaSSGF maintained at 37°C and stirred at 400 rpm for 15 min. The volume was taken down to 50 mL to prevent excessive usage of the simulated fluids. Samples were taken every 5 min. After 15 min, the FaSSGF solution was transferred to 50 mL of double concentrated FaSSIF maintained at 37°C and stirred at 400 rpm. Samples were taken at predetermined time points (30, 60, 90 and 120 min). The pH of FaSSIF upon dilution amounted to 6.5.

Since human intestinal and gastric fluids are scarce and relatively difficult to obtain in comparison to the more readily available simulated fluids, the volumes were reduced for the experiments involving human media. Instead of adding a tablet of Zocor® to a volume of 50 mL, 0.8 mg of SV powder was added to 1 mL of FaHGF maintained at 37°C and stirred at 400 rpm. The addition of 0.8 mg to 1 mL is equivalent to the approach that was used for the simulated fluids, where one tablet of 40 mg simvastatin was added to 50 mL of FaSSGF. Similar proportions were used in both conditions to be able to compare the obtained results. After 15 min, this solution was added to 1 mL of FaHIF maintained at 37°C and stirred at 400 rpm. Samples were taken at predetermined time points (60, 90, 120, and 180 min). The pH of FaHIF decreased from 6.84 to 5.21 upon dilution. All samples were centrifuged (20.817 *g*) and the supernatant was diluted 1/100 in the stabilization mixture before analysis. All experiments were performed in triplicate.

2.6. Clinical trial

To be able to compare the in vitro observations to the in vivo situation, a clinical study was performed. This study included five healthy volunteers (two men, three women) aged between 22 and 26 years. The procedure followed the tenets of the Declaration of Helsinki and was approved by the Committee of Medical Ethics of the University Hospitals Leuven, Belgium (S55581). All volunteers provided written informed consent to participate in this study. After an overnight fast (12 h), two double-lumen polyvinyl catheters [Salem Sump Tube 14 Ch (external diameter 4.7 mm), Sherwood

Medical, Petit Rechain, Belgium] were introduced via the nose and positioned into the stomach and the duodenum (D2/D3). The position of both catheters was checked by fluoroscopy. It has previously been reported that the presence of a transpyloric tube does not influence gastric emptying or duodenogastric reflux. (Müller-Lissner et al., 1982) A single tablet of Zocor® (simvastatin, 40 mg) was administered together with 250 mL of water. Volunteers were asked to sit in upright position in a bed during the sampling procedure. Samples of human gastric and intestinal fluids (sample volume between 1.5 and 4 mL) were aspirated every 10 min for the first hour followed by samples every 15 min up to 4 h. In parallel to the sampling of gastrointestinal fluids, venous blood samples were collected in heparinized tubes (BD Vacutainer systems, Plymouth, UK) at 0, 20, 40, 60, 80, 100, 120, 160, 180, 210, 240, 300, 360, 420 and 480 min after drug intake. These blood samples were centrifuged at 2.880 *g* for 10 min at 4°C to obtain plasma samples which were stored at -30°C until further analysis. In the gastric and intestinal samples, both total amount per volume and the concentration of SV and SVA were measured. For the determination of the total amount per volume, 10 µL of the aspirated fluids was directly diluted 1/100 in stabilization mixture. For the determination of the concentration, the samples were centrifuged (20.817 *g*, 5 min, 37°C) and the supernatant was diluted 1/100 in the stabilization mixture. All samples were stored at -30°C until further analysis.

2.7. Analytical methods

For the quantification of SV and SVA, an LC method with MS/MS detection was developed. All samples contained rosuvastatin as an internal standard at a final concentration of 200 nM. The detection of the 3 compounds was performed using a TSQ Quantum with electron spray ionization. The collision energy for simvastatin, simvastatin acid and rosuvastatin was 27, 33 and 17 V, respectively. Mass transitions for simvastatin, simvastatin acid and rosuvastatin were 441.2/325.1, 435.1/319.1 and 480.2/268.0, respectively. The mass spectrometer was operated in the positive electrospray mode for SV and in the negative electrospray mode for both SVA and RSV. Both compounds SV and SVA were detected using two different instrument methods to avoid overlap in

the electrospray mode. For both methods, the spray voltage, capillary voltage and capillary temperature were 4.50 V, 12 V and 300°C, respectively. Nitrogen was used as the sheath gas (30 arbitrary units), ion sweep (30 arbitrary units) and auxiliary gas (50 arbitrary units). Argon was used as the collision gas at a pressure of 1.5 mTorr. The following gradient of ACN/H_2O/ammonium acetate buffer (pH 4.5) was run over a Kinetex C18 column (50 × 3 mm, 1.7 µm; Phenomenex) protected by a Krudkatcher Ultra HPLC In-Line filter (Phenomenex) at a flow rate of 300 µL/min: 20/72/8 (0–2 min), changed linearly to 95/4/1 (2–2.51 min), keeping the 95/4/1 ratio constant for 1.5 min after which it changed linearly back to the initial conditions of 20/72/8 over 2 min (4.01–6.0 min). The injection volume was 10 µL and Xcalibur was used as the software program (Thermo-Electron, San Jose, USA).

Before quantification of simvastatin and simvastatin acid in plasma by LC-MS/MS, the compounds were extracted from the plasma samples. After diluting 400 µL of plasma in 400 µL of an ammonium acetate solution (100 mM, pH 4.5), 4 µL of internal standard solution in DMSO (RSV, 20 µM) was added. Yang et al. reported that the ammonium acetate buffer minimizes the interconversion between SV and SVA. (Yang et al., 2005) SV and SVA were extracted with 3 mL of diethyl-ether after 10 min of rotatively shaking with a rotary mixer (Labinco 526). After extraction, the organic layer was transferred to a clean test tube and evaporated to dryness under a gentle stream of air. The residue was dissolved in 400 µL of a solution of water and methanol (50:50 v/v), of which 100 µL was injected in the LC-MS/MS system for the detection of each compound.

The calibration curves were linear from 0.9 nM to 1 µM. Precision and accuracy errors determined at 400 nM, 100 nM, 50 nM and 10 nM were below 10%.

2.8. Pharmacokinetic parameters

The pharmacokinetic parameters C_{max}, T_{max}, AUC_{0-8}, $AUC_{0-\infty}$, Cl/F, MRT and Vd_{ss} were calculated using non-compartmental analysis. (Rosenbaum, 2011)

3. RESULTS AND DISCUSSION

Studies discussing the behavior of ester containing compounds in human intestinal fluids have highlighted the impact of intraluminal hydrolysis on intestinal absorption. (Geboers et al., 2015); (Stappaerts et al., 2015) To gain more insight into the disposition of SV upon oral administration, several in vitro experiments reflecting gastrointestinal conditions were performed, including stability and dissolution experiments. The predictive value of these experiments was critically examined by comparing the outcomes of the in vitro studies with the results obtained from a comprehensive in vivo study, in which gastric, duodenal and plasma concentrations were determined.

3.1. Dissolution study

The results of the dissolution study of SV and SVA in both FaHGF and FaHIF are depicted in Figure 2A. After 2 h, the total amount per volume and the concentration of SV and SVA were determined. The 'total amount per volume' refers to the solid and dissolved amount per volume of SV or SVA. 'Concentration' refers to the dissolved amount of SV and SVA per volume. A total amount of 120 µmol of SV per L was measured in FaSSIF and the SV concentration was 11 µM. Moreover, a significant conversion of SV to SVA was observed: since a total amount of 25 µmol of SVA per L was determined of which the solubilized concentration was 4 µM. These results suggest that SV partially degrades to SVA once dissolved in the gastric fluids. The fact that the solubility of SVA in the acidic gastric fluids is lower (pKa SVA = 4.21) than the solubility of SV, results in precipitation of a proportion of the formed SVA.

In the pooled FaHIF (pH 6.84), the concentrations of SV and SVA upon dissolution of SV were measured over a period of 4 h (Figure 2B). The concentrations of SV and SVA that were reached after 4 h of incubation amounted to 148±2 µM and 30±0.2 µM, respectively. This SV concentration is much higher than the solubility reported by Rao et al. in FaSSIF (40 µM). (Rao et al., 2010) This is probably due to the difference in composition of FaSSIF as compared to FaHIF. (Riethorst et al., 2015) Similar

to the gastric fluids, a conversion of SV to SVA was observed. Nevertheless, SV appeared to be more stable in the intestinal fluids than in the gastric fluids.

3.2. Stability study

The dissolution studies already indicated hydrolysis of SV in gastric and intestinal fluids. Since SV is a lactone prodrug, which has to be hydrolyzed in order to attain its active structure, it is important to investigate to which extent gastrointestinal fluids have an impact on the stability of SV. First, the stability of SV and SVA was explored in simulated and human gastric fluids of the fasted state. (Figure 3A) Degradation profiles of SV in both simulated and human gastric fluids indicate that 50% of SV is converted to SVA after 90 min. This is in agreement with literature data which state that SV is unstable below pH 3. (Álvarez-Lueje et al., 2005) In a complementary experiment, the conversion in the opposite direction was also observed upon incubation of SVA in gastric media (data not shown). Considering the relatively low esterase activity in human gastric fluids and the fact that the simulated gastric fluids were not supplemented with hydrolyzing enzymes, the interconversion between SV and SVA is at least partly driven by the acidic pH. (Lund-Pero et al., 1994)

In a next step, the stability of SV was explored in three different intestinal media with levels of complexity increasing in the following order: FaSSIF, FaSSIF supplemented with pancreatin and FaHIF. (Borde et al., 2012); ("U.S. Pharmacopeial Convention," 2009) As compared to the gastric fluids, less degradation was observed (Figure 3B). The degradation of SV to SVA was strongest in FaHIF. As the pH of the simulated (pH = 6.5) and human (pH = 6.8) intestinal fluids was similar, the faster degradation in the more complex media confirms the contribution of enzymatic degradation. The stability of SVA was investigated in these different media as well. However, in contrast to the gastric fluids, the conversion of SVA to SV was negligible (data not shown). Prueksaritanont et al. stated that, in liver homogenates of humans, the hydrolytic rate of conversion of SV to SVA is more than 10-fold higher than in the opposite direction. (Prueksaritanont et al., 2005) The current data suggest that this finding could also hold true in intestinal fluids.

Taking the results of both gastric and intestinal media into account, it can be suggested that the intestinal conversion of SV to SVA is mediated by an enzymatic pathway whereas the gastric conversion is mostly pH driven. Moreover, the conversion of SVA to the inactive lactone form SV was only observed in the gastric fluids and not in the intestinal fluids, suggesting that this reaction is also at least partly mediated by the low pH.

3.3. Two-stage dissolution testing

The in vitro experiments described so far, were designed to evaluate a number of individual physicochemical characteristics of SV, including dissolution, solubility and stability. To gain more insight into the overall gastrointestinal behavior upon oral administration of SV, a more integrated in vitro approach was designed, allowing the simultaneous assessment of dissolution, stability and gastrointestinal transfer effects. In this two-stage dissolution study, gastric and intestinal compartments are connected through a transfer step. Experiments were performed in simulated and human intestinal fluids.

When simulated media were used, one tablet of Zocor® (simvastatin, 40 mg) was added to 50 mL of FaSSGF. The tablet completely disintegrated within 10 min and both SV and SVA were detected in the collected samples of the gastric compartment. After 15 min, the entire content of the vessel was transferred to 50 mL of double concentrated FaSSIF supplemented with double concentrated pancreatin (Figure 4A). The pH of the intestinal compartment upon transfer of the gastric fluids was 6.5. The concentration of SV increased from 14.9 µM in the gastric compartment to 86.4 µM in the intestinal compartment. This is probably due to the solubility enhancing effect of the micelles present in the intestinal media. Data obtained from this two-stage dissolution study were in line with the dissolution and stability studies: (1) relatively slow, incomplete dissolution of SV reaching a concentration of 80 µM in the intestinal compartment after 2 h and (2) more extensive degradation of SV in the gastric fluids than in the intestinal fluids.

For the human media, 0.8 mg of simvastatin was added to 1 mL of FaHGF (pH 1.8). Again, in all gastric samples, both SV and SVA were observed, confirming the simultaneous dissolution and degradation of SV that was observed in the dissolution studies using FaHGF. After 15 min, the entire content was transferred to 1 mL of FaHIF (pH 6.84). Once SV was added to FaHIF, the concentration increased from 7.7 µM to 18 µM (Figure 4B). The increase in concentrations of SV upon transfer was also observed when using simulated intestinal fluids, confirming the solubility enhancing effect of the micelles present in the intestinal fluids. Similar to the two-stage dissolution testing in simulated fluids, hydrolysis of SV occurs faster in the gastric fluids than in the intestinal fluids. In the simulated fluids, concentrations of SV were higher than in the human fluids, both for gastric and intestinal media. This could be due to the presence of solubility or dissolution enhancing excipients in the tablet, such as hypromellose or hydroxypropyl cellulose. (Talukder et al., 2011) Francis et al. showed an increase in solubility of cyclosporine A when hypromellose was included in the formulation. (Francis et al., 2003) These excipients were not included in the two-stage dissolution studies using human intestinal media.

3.4. Clinical study

To date, no intraluminal in vivo data are available which characterize the intestinal behavior of SV. Therefore, a clinical study was performed in which gastric and intestinal fluids were aspirated and analyzed for the total amounts per volume and the concentrations of SV and SVA. In parallel, blood samples were collected to investigate the appearance of SV and SVA in the systemic circulation. A similar approach was already successfully applied to investigate the intestinal behavior of the ester prodrugs fosamprenavir and tenofovir disoproxil fumarate. (Brouwers et al., 2007); (Geboers et al., 2015)

3.4.1. Plasma concentration-time profiles

Although our study mainly focused on the intestinal behavior of SV, blood sampling allows validating our results in view of earlier performed clinical studies involving oral administration of SV. Both SV

and SVA were observed in the plasma samples. (Figure 5) Prueksaritanont et al. studied the complex metabolism of SV and SVA, which are both CYP3A4 substrates but also undergo interconversion in vivo. (Prueksaritanont et al., 2003) The pharmacokinetic parameters of both compounds are listed in Table 1. The C_{max} of SVA (3.90 nM) is reached 140 min later than the C_{max} of SV (23.3 nM). Similar C_{max} and T_{max} values were reported in literature. (Backman, 2000) The $AUC_{0\text{-}8h}$ of SV (36.10^2 nM.min) is 3.4 times higher than the $AUC_{0\text{-}8h}$ of SVA (11.10^2 nM.min). Based on the $AUC_{0\text{-}\infty}$, the mean residence time (MRT) of SV was found to be 1.55 h.

3.4.2. Gastrointestinal concentration-time profiles

In parallel to the blood sampling, gastric and intestinal fluids were collected to investigate the intraluminal behavior of SV. Gastric and intestinal fluids were collected at predetermined time points and analyzed for the total amount per volume and concentrations of SV and SVA.

3.4.2.1. Gastric concentration-time profiles

Figure 6 shows the concentration-time profiles of SV and SVA in the stomach upon administration of one tablet of Zocor® (simvastatin, 40 mg). The concentrations (A) and total amount per liter (B) of SV and SVA are depicted. The pharmacokinetic parameters are shown in Table 2. The concentration-time profiles of SV and SVA reach their T_{max} value 44±15 min and 65±19 min, respectively, after drug administration, both reaching a C_{max} of 3±3 µM. However, the total amount of SV and SVA per liter, are 75 and 19 times higher, respectively, than the concentration found in the stomach of both compounds. This finding is an in vivo confirmation of the poor dissolution and solubility characteristics that were observed for SV in gastric fluids in the dissolution study (Figure 2). Moreover, the two-stage dissolution test already predicted the concentrations of SV and SVA to be fairly similar in the stomach (Figure 4). These gastric concentrations reached in vivo remain somewhat lower than the maximum concentration that was measured during the performed dissolution study (Figure 2). (Rao et al., 2010) This can be explained by the continuous process of gastric emptying, keeping the concentrations of SV and SVA low. SVA exhibits a poor solubility (pKa

SVA = 4.21) in the low pH ranges of the aspirated gastric media. Based on the high T_{max} value and the low concentrations of both SV and SVA, it can be concluded that SV dissolves slowly in the stomach, followed by conversion of SV to SVA. Subsequent precipitation of SVA results from its inferior solubility in gastric media as compared to SV.

3.4.2.2. Intestinal concentration-time profiles

Figure 7 depicts the duodenal concentration-time profiles of SV and SVA after the intake of one tablet of Zocor® (simvastatin, 40 mg); the concentrations (A) and the total duodenal amounts per liter (B) of SV and SVA are shown. The pharmacokinetic parameters, calculated based on the individual volunteers, are shown in Table 2. Both C_{max} and $AUC_{0\text{-}4h}$ of the total amounts per liter of SV and SVA are lower in the intestine than in the stomach. In addition to the dilution that takes place upon transfer, this could also be caused by fast absorption of dissolved SV and SVA in the intestine. Both SV and SVA exhibit higher solubility in the intestine than in the acidic environment of the stomach, increasing the concentration gradient of SV and SVA across the intestinal layer, favoring the intestinal uptake of both compounds. The superior solubility and dissolution characteristics of SV in the intestinal fluids were evident from the in vitro dissolution experiments (Figure 2). Based on the obtained gastric and intestinal concentration-time profiles it can be concluded that dissolution of SV in the stomach is slow, followed by rapid conversion to the less soluble SVA, whereas in the intestine the superior dissolution rate, solubility and stability of SV leads to higher concentrations of solubilized SV. On the other hand, the concentrations of SVA at the level of the duodenum remain low as compared to the SV concentrations. This is probably due to the slow dissolution of SVA in intestinal fluids (Figure 2B) and the relatively good stability of SV in intestinal fluids (Figure 3B). Given the fact that intestinal absorption of SV (and SVA) is mostly dissolution limited, compound solubilized in the small intestine will be rapidly taken up across the intestinal barrier.

When comparing the in vivo results with the results obtained during the two-stage dissolution experiments, similar findings were observed. SV slowly dissolves in FaHGF, but rapidly converts to

SVA. A fast degradation of SV to SVA was observed in the clinical study as well. (Table 2 and Figure 3) In the in vitro two-stage dissolution study using human intestinal fluids, concentrations of SV and SVA were similar as observed in the in vivo study. Moreover, the slow in vivo dissolution of SVA as compared to SV was also clearly reflected in the two-stage dissolution study. These findings underline the predictive and added value of this relatively simple in vitro experiment. Due to the simplicity and the good in vivo predictability of this in vitro experiment, it is warranted to explore the applicability of the two-stage dissolution tests for other API's or formulations.

Although, similar as in plasma, concentrations of SV in the small intestine are higher than those of SVA, it remains difficult to directly correlate the intestinal concentration profiles to the plasma profiles. Since no correlation could be found between the observed plasma and intraluminal concentrations, it can be assumed that the gut wall and hepatic metabolism have a major impact on the oral bioavailability of simvastatin and outweigh the importance of the intraluminal behavior of simvastatin. (Gertz et al., 2010)

4. CONCLUSION

Based on the in vitro experiments, it could be concluded that (1) the dissolution of SV is slow; (2) the hydrolysis of SV to SVA occurs both in gastric and intestinal fluids while the lactone-hydroxyl acid interconversion was only observed in the gastric fluids and (3) the hydrolysis rate is higher in gastric fluids than in intestinal fluids, suggesting an important contribution of non-enzymatic hydrolysis to the overall intraluminal degradation of SV. By increasing the complexity of the in vitro set-ups, results more predictive for the in vivo situation were obtained. Despite the fact that, in case of SV, gut wall and hepatic metabolism outweigh the importance of the intraluminal behavior, the in vitro tools studied here can be useful to predict the gastrointestinal behavior of a prodrug.

5. FIGURE LEGENDS

Figure 1: Metabolism of simvastatin in man. (adopted from(Pedersen and Tobert, 2004); (Prueksaritanont, 2002))

Figure 2: [A] Concentration-time profile of the dissolved amount of SV (●) and SVA (■) during the dissolution experiment in which 0.13 mg SV powder was added to 1 mL of FaHGF (175 rpm, 37 °C); [B] Concentration-time profile of the dissolved amount of SV (●) and SVA (■) during the dissolution experiment in which 0.13 mg of SV powder was added to 1 mL of FaHIF (175 rpm, 37°C). (Mean±SD, n = 3))

Figure 3: Stability of 10 µM SV (solid lines) in fasted state [A] gastric and [B] intestinal fluids in which SV degrades to SVA (dotted lines) after a period of time. (●) simulated gastric/intestinal fluids; (■) simulated intestinal fluids supplemented with 10 mg pancreatin per mL; (▲) human gastric and intestinal fluids in fasted state. (Mean±SD, n = 3)

Figure 4: Concentration-time profile of SV (●) and SVA (■) during the two-stage dissolution testing. (A) The addition of one tablet of Zocor® (simvastatin, 40 mg) to simulated fluids and (B) the addition of 0.8 mg of SV to 1 mL of human fluids. (Mean±SD, n = 3)

Figure 5: Plasma-concentration-time profile of SV (●) and SVA (■) in the fasted state condition after the administration of one tablet of Zocor® (simvastatin, 40 mg) to healthy volunteers. (Mean±SEM, n = 5)

Figure 6: Concentration-time profile of SV (●) and SVA (■) in the stomach after the intake of one tablet of Zocor® (simvastatin, 40 mg) by healthy volunteers in the fasted state condition. (A) Concentration-time profile of the dissolved amount. (B) Concentration-time profile of the total amount. (Mean+SEM, n = 5)

Figure 7: Intestinal concentration-time profiles of SV (●) and SVA (■) after the intake of one tablet of Zocor® (simvastatin, 40 mg) by healthy volunteers in the fasted state condition. (A) Concentration-time profile of the dissolved amount. (B) Concentration-time profile of the total amount. (Mean+SEM, n = 5)

Table 1: The clinical pharmacokinetic parameters of SV and SVA in the fasted state after the administration of one tablet of Zocor® (simvastatin, 40 mg) to five healthy volunteers. (Median [min, max], n = 5)

Table 2: The pharmacokinetic parameters, calculated based on the individual volunteers, of SV and SVA in both stomach and intestinal samples after the administration of one tablet of Zocor (simvastatin, 40 mg) to five healthy volunteers. (Mean±SD, n=5)

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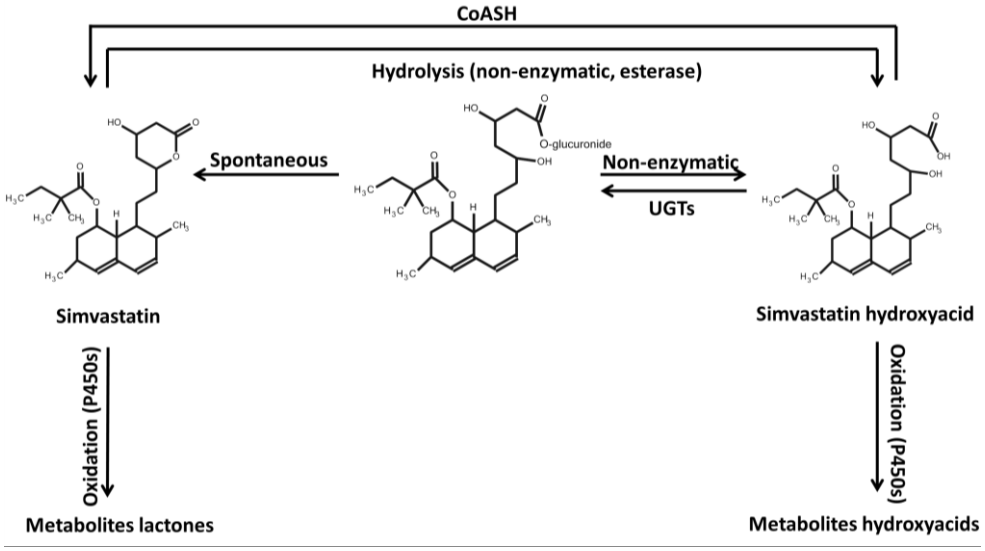
7. Figures and tables

Figure 1:



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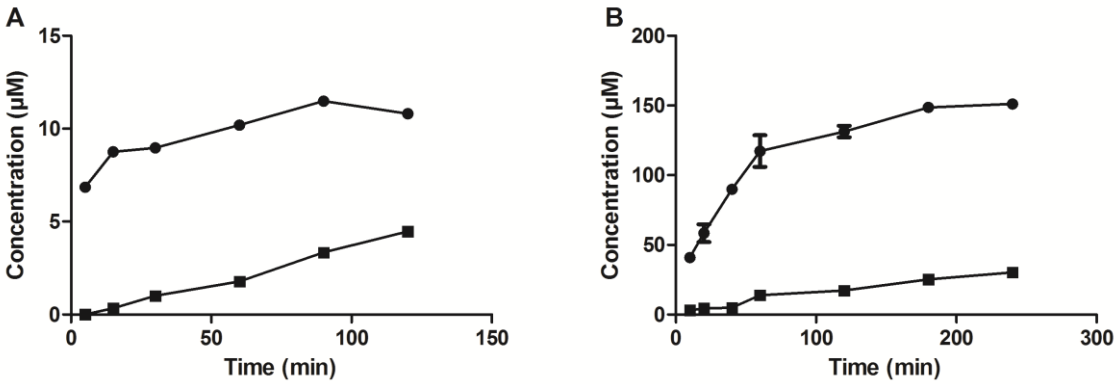

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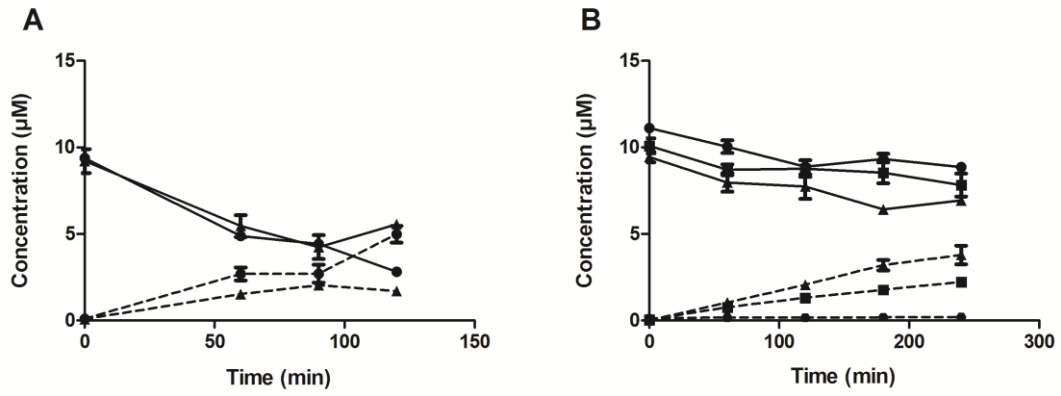

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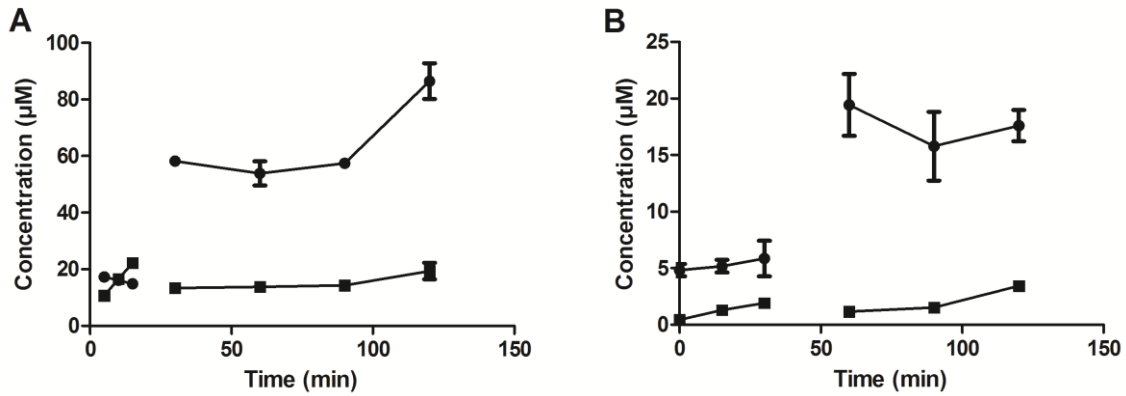

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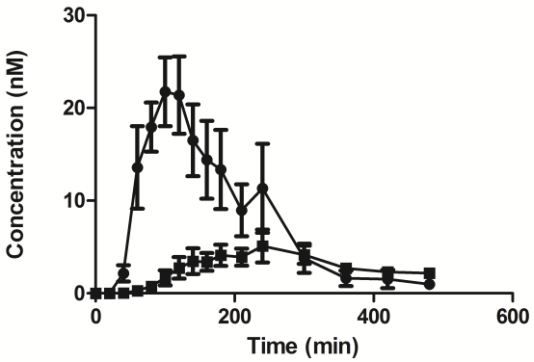

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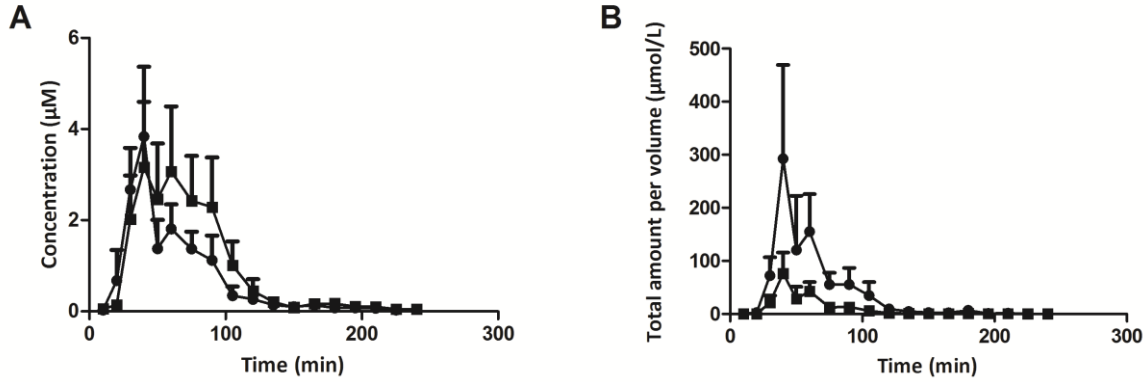

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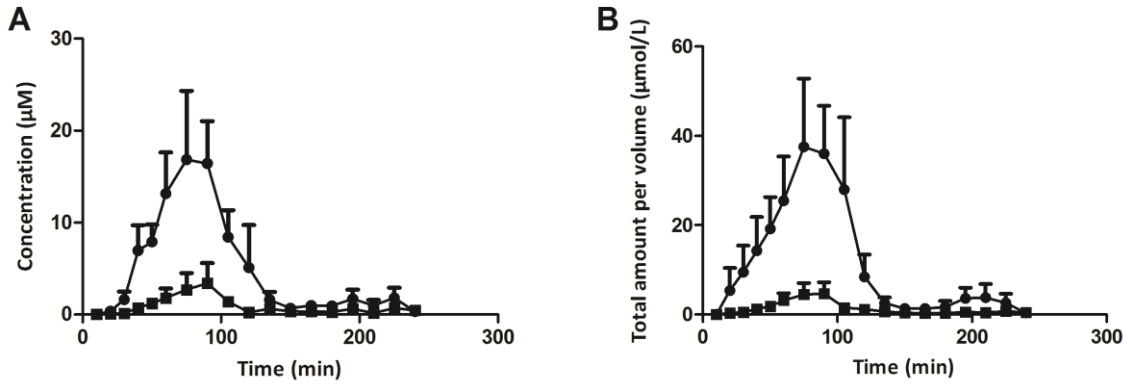

Table 1:

	Simvastatin (SV)	Simvastatin acid (SVA)
C_{max} (nM)	23.3 [10.5-31.1]	3.90 [3.21-12.2]
T_{max} (min)	99.6 [60.0-120]	240[210-300]
AUC_{0-8} (nM.min)	3572 [663-6069]	11.10^2 [$8.3.10^2$-27.10^2]
$AUC_{0-\infty}$ (nM.min)[a]	3572 [663-6509]	-[a]
Cl/F (L/h)	1698 [881-8646]	-[a]
MRT (h)	1.55 [0.83-2.12]	-[a]
Vd_{ss} (Cl/k)	1868 [1627-7138]	-[a]

[a] Parameters could not be calculated due to an incorrect extrapolation of the $AUC_{8-\infty}$.

Table 2:

	Simvastatin (SV)		Simvastatin acid (SVA)	
	Stomach	**Intestine**	**Stomach**	**Intestine**
$C_{max,\ dissolved}$ (µM)	3.3±3.1	23±11	3.5±3.4	3.8±4.6
$T_{max,\ dissolved}$ (min)	44±15	78±13	65±19	84±17
$AUC_{0\text{-}4h,\ dissolved}$ (µM.min)	270±150	1200±430	91±58	220±180
$C_{max,\ total}$ (µmol/L)	250±320	60±31	68±73	5.6±5.3
$T_{max,\ total}$ (min)	40±12	71±23	40±12	87±22
$AUC_{0\text{-}4h,\ total}$ (µmol/L.min)	9500±12000	2900±1500	2400±2500	320±240